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Evolution

Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*

(evolution/rRNA/phylogeny/random amplified polymorphic DNA/cellulase producers)

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ABSTRACT **The relationship of the important cellulase producing asexual fungus *Trichoderma reesei* to its putative teleomorphic (sexual) ancestor *Hypocrea jecorina* and other species of the *Trichoderma* sect. *Longibrachiatum* was studied by PCR-fingerprinting and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene. The differences in the corresponding ITS sequences allowed a grouping of anamorphic (asexual) species of *Trichoderma* sect. *Longibrachiatum* into *Trichoderma longibrachiatum*, *Trichoderma pseudokoningii*, and *Trichoderma reesei*. The sexual species *Hypocrea schweinitzii* and *H. jecorina* were also clearly separated from each other. *H. jecorina* and *T. reesei* exhibited identical sequences, suggesting close relatedness or even species identity. Intraspecific and interspecific variation in the PCR-fingerprinting patterns supported the differentiation of species based on ITS sequences, the grouping of the strains, and the assignment of these strains to individual species. The variations between *T. reesei* and *H. jecorina* were at the same order of magnitude as found between all strains of *H. jecorina*, but much lower than the observed interspecific variations. Identical ITS sequences and the high similarity of PCR-fingerprinting patterns indicate a very close relationship between *T. reesei* and *H. jecorina*, whereas differences of the ITS sequences and the PCR-fingerprinting patterns show a clear phylogenetic distance between *T. reesei*/*H. jecorina* and *T. longibrachiatum*. *T. reesei* is considered to be an asexual, clonal line derived from a population of the tropical ascomycete *H. jecorina*.**

Trichoderma reesei is an asexually reproducing filamentous fungus. Isolated in the Solomon Islands during World War II from cotton canvas, the species is unique because it is known only from a single isolate (QM6a). This isolate is renowned for its high ability to produce cellulases and it is the sole progenitor of the many mutants currently in use (1). *T. reesei* is not known to undergo recombination through meiosis. Many deuteromycetes are permanently sexually incompetent (2, 3) and possibly clonal derivatives of life cycles that once included a meiosis, some aspect of an outcrossing mating system having been perturbed. Because *Trichoderma* is a genus of special economic interest—individual species are used in biological control (4, 5), in the production of cellulolytic and other hydrolytic enzymes (6, 7), and in antibiotics (8)—the identification of a teleomorph (sexual state) would be useful. The ability to recover meiotic progeny would provide a means to study biological properties using classical genetics, an approach that has proven invaluable in the study of the molecular biology of *Neurospora crassa* and *Aspergillus nidulans* (9). Further, teleomorphs are powerful predictors of taxonomic relationships, and related species can be expected to possess similar biological abilities. The question thus arises whether *T. reesei*, and many other fungi of economic importance, has a teleomorph and, if so, how it could be recognized.

The system of deuteromycetes (asexual fungi) is based on form, and species are recognized because they are perceived to be morphologically different from the type species of the genus to which they are assigned. However, the degree of morphological similarity does not necessarily correlate with phylogenetic relatedness. Thus, the taxonomy of deuteromycetes has always been deliberately artificial (10). In the case of the genus *Trichoderma*, species are difficult to distinguish and a correlation with teleomorphic species on morphological basis is often not possible (11).

T. reesei represents an example of our inability to predict relationships based upon morphology alone. *T. reesei* was initially distinguished from *Trichoderma viride* Pers.:Fr. and *Trichoderma longibrachiatum* Rifai both on the basis of its unusually high production of cellulolytic enzymes and on its morphological characteristics (12). Considering only morphology, Bissett (13) synonymized *T. reesei* under *T. longibrachiatum*. However, restriction fragment length polymorphism methods (14, 15) and combined morphometric and isozyme analyses (16, 17) show that *T. reesei* and *T. longibrachiatum* are taxonomically separable. Moreover, considerable morphological and isozyme differences between temperate and tropical collections of *Hypocrea schweinitzii* led to the recognition that the tropical collections were actually *Hypocrea jecorina* Berk. & Broome. It was also shown that *T. reesei* was more closely related to *Hypocrea jecorina* than either fungus is to *H. schweinitzii*, *T. longibrachiatum*, or the other species of sect. *Longibrachiatum* (16).

DNA sequencing has been useful in confirming links, postulated by more classical observations, between anamorphs and teleomorphs at generic and higher level (18–21). We analyzed DNA of *T. reesei* and related strains and found a link at the species level. We will show that *T. reesei* and *H. jecorina*

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Abbreviation: ITS, internal transcribed spacer.
Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X93931–X93936, X93950–X93956, X93960, X93962, X93975, X93978, X93979, X93986, Z31013, Z31014, Z31016, Z31018, Z31019, Z48811, Z48812, Z48932–Z48935, Z48948).
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have identical nucleotide sequences in their internal transcribed spacer (ITS) regions of the ribosomal DNA and exhibit similar patterns of polymerase chain reaction (PCR)-fingerprints, which was found to be typical only for strains of the same species. We therefore consider *T. reesei* to be a clonal derivative of *H. jecorina*. The molecular relationship of *T. reesei* to *H. jecorina* suggests an attractive model for the investigation of the process of speciation in deuteromycetes while using an organism of industrial importance.

MATERIALS AND METHODS

Fungal Cultures. Sources and designation of the *Trichoderma* and *Hypocrea* strains are listed in Table 1. Only those strains that were confirmed by additional morphological studies to have been correctly identified were used. Fungal cultures were grown as described (23).

DNA Isolation, PCR-Fingerprinting Assay, and Hybridization. DNA was isolated as described (23). PCR-fingerprinting reactions were carried out as described (23–25). Five radioactively labeled fragments of a PCR-fingerprint with primer M13, characteristic for both *T. reesei* and *H. jecorina*, were used as probes for hybridization with the Southern blot of the same M13 PCR-fingerprint. Labeling reaction was performed using the Rediprime DNA labeling system (Amersham) according to the manufacturer's protocol. Hybridization was performed according to standard methods (26).

rDNA Amplification Assay. Amplification of rDNA was performed as described by Vilgalys and Hester (27). We used primers that consist of a conservative sequence within the small subunit rDNA (primer SR6R), the 5.8S rDNA (primers

Table 1. List of investigated fungal strains

Species/strain	Origin	Geographic origin	ITS sequence class*	GenBank accession no.
T. reesei QM6a ex type	ATCC 13631=DAOM 167654	Bougainville Island	A	Z31016
T. reesei QM9123	ATCC 24449	Mutant of QM6a	A	Z48933
T. reesei QM9414	ATCC 26921	Mutant of QM9123	A	Z48933
T. reesei QM6c	IMI 045548		—	—
T. reesei†	IMI 289235	Singapore	A	Z48932
T. longibrachiatum ex type	ATCC 18648=CBS 816.68	Ohio	B1	Z31019
T. longibrachiatum‡	ATCC 60641=CBS 389.92	Ecuador	B1	Z48934
T. longibrachiatum‡	ATCC 38586=CBS 397.92	Not indicated	B1	Z48948
T. longibrachiatum	IMI 287096	India	B1	X93935
T. longibrachiatum	CBS 489.78	Colombia	B1	X93934
T. longibrachiatum	ATCC 52326	India	B2	Z48935
T. longibrachiatum	DAOM 166989	Canada	B2	X93931
T. longibrachiatum	TR 87	Ohio	B2	X93936
T. longibrachiatum	PJS 79-3	Austria	B2	X93932
T. longibrachiatum	GJS 91-157	Switzerland	B3	X93933
Trichoderma pseudokoningii ex type	DAOM 167678=CBS 408.91	Australia	C	Z31014
H. jecorina	CBS 822.91	Indonesia	A	Z31018
H. jecorina	GJS 85-249	Indonesia	A	X93950
H. jecorina	CBS 836.91	Brazil	A	X93951
H. jecorina	GJS 86-403	French Guiana	—	—
H. jecorina	GJS 86-404	French Guiana	—	—
H. jecorina	GJS 86-408	French Guiana	—	—
H. jecorina	CBS 815.91	French Guiana	A	X93952
H. jecorina	CBS 816.91	French Guiana	A	Z31018
H. jecorina	GJS 84-473	Venezuela	—	—
H. jecorina	CTR 72-94	Venezuela	—	—
H. jecorina	GJS 93-19	New Caledonia	A	X93953
H. jecorina	GJS 91-22	New Caledonia	A	X93954
H. jecorina	GJS 91-23	New Caledonia	A	X93955
H. jecorina	GJS 91-24	New Caledonia	A	X93956
H. schweinitzii	CBS 817.91	USA	D	X93960
H. schweinitzii	CBS 818.91	USA	D	Z31013
H. schweinitzii	GJS 93-1	USA	D	X93962
Hypocrea ?aurantia	GJS 91-141	USA	—	—
T. viride	CBS 240.63	U.K.	F	X93979
T. viride	ATCC 18652=TR2	Not indicated	F	X93978
T. viride	TR8	Not indicated	F	X93986
Trichoderma harzianum/atroviride‡	ATCC 32173=CBS 396.92	Israel	G	Z48811
T. harzianum/atroviride‡	ATCC 42831=CBS 398.92	Hungary	G	Z48811
T. harzianum/atroviride‡	ATCC 36042=CBS 391.92	Not indicated	G	Z48812
Trichoderma hamatum‡	ATCC 28012=CBS 393.92	USA	H	X93975

ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; DAOM, Department of Agriculture (Mycology), Ottawa, Canada; IMI, Commonwealth Agricultural Bureau International Mycological Institute, Eggham, U.K.; TR, see Meyer and Plaskowitz (22); GJS, collection of G.J.S.; CTR, collection of C. T. Rogerson (New York Botanical Garden, Bronx, NY).

*Sequences marked with the same letter are identical and letters with numbers are sequences that differ in 1-2 base pairs, presenting subgroups within a species.

†Appears in the IMI catalogue under the name *T. longibrachiatum*. The indicated geographic origin is incorrect; this strain originally was obtained from U.S. Army Natick Research and Development Laboratories (Natick, MA) and is actually *T. reesei* QM6a.

‡These strains appear in the ATCC catalogue under a different name. The names in the list were redetermined by W.G.

5.8S and 5.8SR), and the large subunit rDNA (primer LR1), flanking the ITSs (ITS-1 and ITS-2) (27).

DNA Sequencing. Amplified rDNA fragments were purified before sequencing by use of PCR Preps Magic Columns (Promega). Purified double stranded PCR fragments were sequenced by direct cycle sequencing following the protocol supplied with the Cycle Sequencing System (GIBCO/BRL). One picomole of the respective sequencing primer (SR6R, 5.8S, 5.8SR, LR1) was end-labeled with [γ-^{33}P]ATP. Cycle sequencing was performed in a Hybaid (Middlesex, U.K.) thermocycler programmed for an initial denaturation step of 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, 2 min at 70°C. Sequencing reactions were run on 8% polyacrylamid/TBE-urea gels.

Data Analysis. Sequences were initially aligned using the multiple alignment program CLUSTAL V at the European Molecular Biology Laboratory (Heidelberg). The alignment was optimized visually when needed (alignment is available upon request). Phylogenetic analysis was performed using parsimony (PAUP 3.0) (28). Single gaps were treated as missing characters. Sequences of *T. viride*, *T. harzianum/atroviride* (sect. *Trichoderma*), and *T. hamatum* (sect. *Pachybasium*) were used as outgroup. The search for the most parsimonious trees was carried out in two stages. The first analysis included all sequences and was performed using the heuristic search option. A second search was performed on a subset of the eight recognizable sequence-types using the branch-and-bound algorithm. The robustness of internal branches was tested by bootstrap analysis (29) from 1,000 bootstrap replications using the heuristic search option.

Analysis of the PCR-fingerprints was carried out by pairwise comparison of the patterns of all strains for every primer. All amplified DNA-fragments that reproducibly occurred were scored based on two possible character states: 0 (fragment absent) and 1 (fragment present). Both parsimony analysis (using PAUP 3.0) and phenetic analyses with the unweighted pair group method using arithmetic averages (UPGMA) and the Fitch–Margoliash method (30, 31) offered by the program package PHYLIP 3.4 (31) were carried out. A pairwise distance matrix was calculated with the distance index proposed by Nei and Li (32): $D_{xy} = 1 - 2N_{xy}/(N_x + N_y)$, in which N_{xy} is the number of fragments shared between a pair of strains, and N_x and N_y are the numbers of fragments present in strain x and y, respectively. The 0/1 character matrices and matrices of D values are available on request. Average inter- and intraspecies similarities ($S = 1 - D$) were used to assess the degree of variation at different taxonomical levels.

RESULTS

Sequencing of ITS-1 and ITS-2 of the rDNA gene complex was undertaken because these regions are known to be highly variable (33, 34) and suitable for phylogenetic studies of fungi at the inter- and intraspecific level (35–39). Both strands of a fragment containing ITS-1, 5.8S, and ITS-2 were sequenced. The length of the corresponding fragments was 619–652 bp, also containing a part of the 3′-end of the 18S rDNA and the 5′-end of the 28S rDNA. ITS-1 showed a considerably higher variability than ITS-2 (Table 2).

Each species (13) has a characteristic ITS-sequence type. Intraspecific variation could not be detected despite the often very different geographic origins of the strains. The only exception to this rule was found for *T. longibrachiatum* with two nucleotide exchanges in ITS-1 (0.9%), dividing this species into three subgroups. The sequence of all strains of *T. reesei* differed from those of *T. longibrachiatum* in 6 bp (2.6%) for ITS-1 and 1 bp (0.6%) for ITS-2. Identical sequences were found also for all strains of *H. jecorina.* The ITS sequences of all strains of *H. schweinitzii* collected from temperate geographical sites were identical and differed from those of *H. jecorina* in 5 bp (2.2%) in ITS-1. The ex type strain (i.e., the strain derived from the dead herbarium reference collection for a species) of *T. pseudokoningii* also exhibited a unique sequence, which was not shared by any other species of the section (Table 2). Interestingly, the sequences of the ascomycete *H. jecorina* and the deuteromycete *T. reesei* were identical. In our continuing study of *Trichoderma*, different species did not share identical ITS-sequences (unpublished work).

Table 2. Intra- and interspecific variation within ITS-1 and ITS-2 of the investigated fungal strains

Compared isolates/taxonomic level	ITS-1 No. of diff. bp.	ITS-1 V (%)	ITS-2 No. of diff. bp	ITS-2 V (%)
Intraspecies				
Tr (4)	0	0.0	0	0.0
Hj (9)	0	0.0	0	0.0
Tl (13)	2	0.9	0	0.0
Hs (3)	0	0.0	0	0.0
Tv (4)	0	0.0	0	0.0
Interspecies				
Tr-Hj (4/9) (intraspecies?)	0	0.0	0	0.0
Tr-Tl (4/10)	6	2.6	1	0.6
Tr-Tp (4/1)	11	4.8	1	0.6
Tr-Hs (4/3)	5	2.2	0	0.0
Tl-Tp (10/1)	11	4.8	2	1.1
Tl-Hs (10/3)	7	3.1	1	0.6
Tl-Hj (10/9)	6	2.6	1	0.6
Tp-Hs (1/3)	6	2.6	1	0.6
Tp-Hj (1/9)	11	4.8	1	0.6
Hs-Hj (3/9)	5	2.2	0	0.0
Intersection				
Tl-Tv (10/4)	73	32.0	24	13.4
Tl-Thz (10/3)	71	31.1	21	11.7
Tl-Thm (10/1)	46	20.2	13	7.3

V, variation of the sequences expressed in percent; Tr, *T. reesei*; Tl, *T. longibrachiatum*; Tp, *T. pseudokoningii*; Hj, *H. jecorina*; Hs, *H. schweinitzii*; Tv, *T. viride*; Thz, *T. harzianum/atroviride*.; Thm, *T. hamatum*.
The value in brackets indicates the number of strains compared.

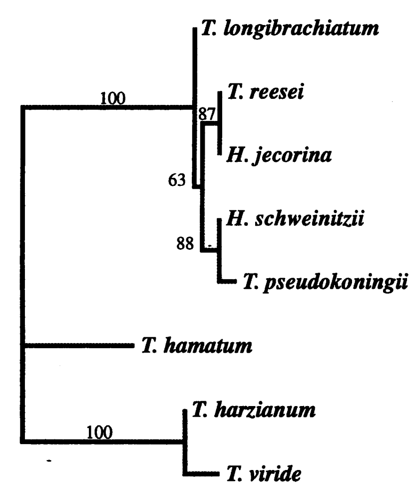

FIG. 1. Phylogenetic relationships of the investigated *Trichoderma* and *Hypocrea* species. The phylogram is based on rDNA sequences including the ITS-1, 5.8S, and ITS-2 regions and presents the most parsimonious tree generated from the branch-and-bound algorithm in PAUP 3.0. The search was performed on the eight recognizable sequence types, representing species-specific sequences. *T. hamatum*, *T. viride*, and *T. harzianum/atroviride* were used as outgroup. The length of the tree was 63 steps, and the consistency index was 1.0. Percentages above the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications.

The ITS-sequence type for each strain is indicated in Table 1. Table 2 illustrates the variation in the ITS-1 and ITS-2 sequences within and between the investigated species. Fig. 1 presents a phylogenetic tree obtained by parsimony analysis of the ITS sequences of the species assignable to *Trichoderma* sect. *Longibrachiatum*. Species from sect. *Trichoderma* (*T. viride* and *T. harzianum/atroviride*) and sect. *Pachybasium* (*T. hamatum*) were included as outgroup. This tree is based on the eight different sequence types found. Low variation within *Trichoderma* sect. *Longibrachiatum* is apparent. Nucleotide variation among the most distantly related species was 4.8% for ITS-1 and 1.1% for ITS-2, making it debatable whether grouping into species within the section is justified.

The identical rDNA sequence in *T. reesei* and *H. jecorina* could be an indication that both fungi represent the same species. To test whether *T. reesei* actually falls into the genetic variation of *H. jecorina*, PCR-fingerprinting was performed with DNA of all strains using three different primers [$(GACA)_4$, $(GTG)_5$, and M13 core sequence]. These primers previously were found to be useful in analysis of fungi at the species level and even for strain identification (23–25). The patterns observed were reproducible and indicated the same relationships between the strains when carried out in two different laboratories. An example of the PCR-fingerprints is shown in Fig. 2*A*. Interestingly, the ex type strain of *T. reesei* and its mutants were indistinguishable from each other. Obviously the PCR-fingerprint pattern remained stable over the years of independent cultivation of the strains, because all originated from one and the same isolate.

H. jecorina PCR-fingerprinting patterns closely resemble those of *T. reesei*. Variation among different strains of *H. jecorina* was generally in the range of 18–24%, indicating relatively little variation in its genetic pool despite its wide geographic distribution (Indonesia, New Caledonia, Venezuela, Brazil, French Guiana). Variation between *T. reesei* and *H. jecorina* was essentially of the same order of magnitude (18–25%) as the intraspecific variation within *H. jecorina*. This supports the conclusion that *T. reesei* behaves like any other strain of *H. jecorina*. Genetic variation within *T. longibrachiatum* was slightly higher (27–39%). Table 3 shows the average inter- and intraspecies similarities calculated for each primer. Intraspecies variabilities varied generally between 0–39%,

Table 3. Average similarities at intra- and interspecies level calculated from PCR-fingerprinting patterns obtained by three different primers

Compared isolates/taxonomic level	Average similarity (S)		
	Primer $(GTG)_5$ S (d)	Primer $(GACA)_4$ S (d)	Primer M13 S (d)
Intraspecies			
Tr (4)	1.00 (±0.000)	1.00 (±0.000)	1.00 (±0.000)
Hj (4)	0.76 (±0.047)	0.79 (±0.106)	0.82 (±0.097)
Tl (3)	0.83 (±0.109)	0.68 (±0.124)	0.61 (±0.121)
Hs (2)	0.71 (±0.000)	0.44 (±0.000)	0.63 (±0.000)
Interspecies			
Tr-Hj (intraspecies?)	0.75 (±0.033)	0.82 (±0.047)	0.81 (±0.057)
Tr-Tl	0.40 (±0.031)	0.30 (±0.052)	0.16 (±0.027)
Tr-Tp	0.25 (±0.000)	0.11 (±0.000)	0.26 (±0.000)
Tr-Hs	0.18 (±0.013)	0.35 (±0.082)	0.26 (±0.030)
Tl-Tp	0.28 (±0.073)	0.13 (±0.058)	0.30 (±0.051)
Tl-Hj	0.43 (±0.030)	0.28 (±0.068)	0.19 (±0.055)
Tl-Hs	0.17 (±0.026)	0.23 (±0.025)	0.15 (±0.125)
Tp-Hj	0.18 (±0.047)	0.16 (±0.046)	0.17 (±0.040)
Tp-Hs	0.30 (±0.042)	0.35 (±0.075)	0.33 (±0.000)
Hj-Hs	0.16 (±0.032)	0.36 (±0.118)	0.15 (±0.031)

S, average similarity; d, average deviation from the average similarity value; Tr, *T. reesei*; Tl, *T. longibrachiatum*; Tp, *T. pseudokoningii*, Hj, *H. jecorina*; Hs, *H. schweinitzii*. The value in brackets indicates the number of strains compared.

whereas interspecies variabilities were between 57–89%. The variabilities between *T. reesei* and *H. jecorina* were in the range found for the intraspecies level, whereas the variability between *T. reesei* and *T. longibrachiatum* was as observed for the interspecies level.

The same variation in values specific for the intra- and interspecies level were found also when more species and a larger set of strains (10–25 per species) was investigated (data not shown; unpublished work). These values were consistent independent of the primer used, which indicates little, if any, influence on the results of comigration of bands bearing different sequences. To prove homology of fragments with identical electrophoretic mobility, shared fragments characteristic for *T. reesei* and *H. jecorina* were isolated from the gel.

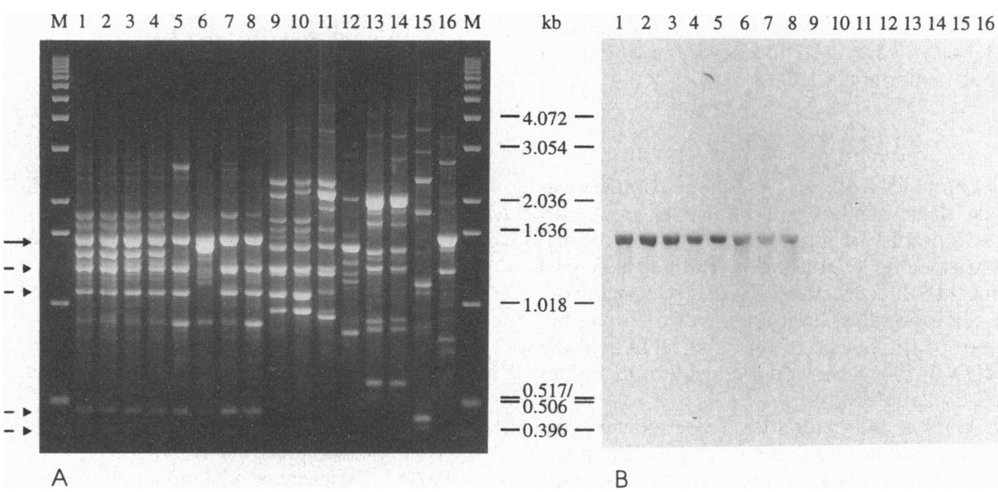

FIG. 2. (*A*) PCR-fingerprinting patterns obtained by amplification of genomic DNA from strains of *Trichoderma* and *Hypocrea* with phage M13 core sequence primer. Lanes: 1, *T. reesei* QM6a ATCC 13631; 2, *T. reesei* QM9414 ATCC 26921; 3, *T. reesei* QM6c IMI 045548; 4, *T. reesei* IMI 289235; 5, *H. jecorina* CBS 815.91; 6, *H. jecorina* CBS 822.91; 7, *H. jecorina* CBS 836.91; 8, *H. jecorina* GJS 86-403; 9, *T. longibrachiatum* ATCC 18648; 10, *T. longibrachiatum* ATCC 60641; 11, *T. longibrachiatum* ATCC 52326; 12, *T. pseudokoningii* DAOM 167678; 13, *H. schweinitzii* CBS 817.91; 14, *H. schweinitzii* CBS 818.91; 15, *H. ?aurantia* GJS 91-141; 16, *T. viride* ATCC 18652. M, molecular weight marker (1 kb ladder). (*B*) Southern blot hybridization of the M13 PCR-fingerprint shown in *A* probed with a fragment of the M13 PCR-fingerprint characteristic for both *T. reesei* and *H. jecorina* as indicated by the solid arrow in *A*. Four additional characteristic fragments, indicated by dotted arrows, were used as probes, showing strong hybridization signals for both *T. reesei* and *H. jecorina*.

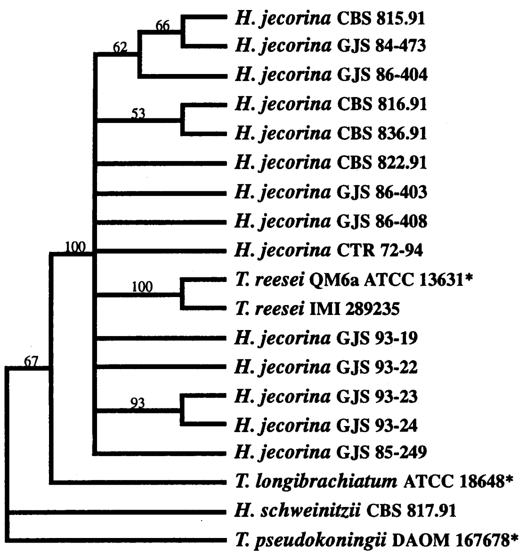

FIG. 3. Cladogram inferred by parsimony analysis with PAUP 3.0 based on the PCR-fingerprints with primers $(GACA)_4$, $(GTG)_5$, and M13 of 14 isolates of *H. jecorina*, *T. reesei* QM6a, *H. schweinitzii* CBS 817.91, and the ex type strains of *T. longibrachiatum* and *T. pseudokoningii* (PCR-fingerprints not shown). Ex type strains are marked by an asterisk. The presented tree is the strict consensus of 33 most parsimonious trees found. *T. pseudokoningii* DAOM 167678 was used as outgroup for tree reconstruction. Values above the branches indicate the percentages with which the branches appeared in 500 bootstrap replications.

These fragments were used as probes for hybridization with the Southern blot of the PCR-fingerprinting gel. An equally strong signal at the same position was observed for the *H. jecorina* strains as well as for all four *T. reesei* strains, indicating strong sequence homology for the respective fragments (see Fig. 2*B* for an example).

To demonstrate the variability among *H. jecorina* strains and the position of *T. reesei* within this *H. jecorina* cluster, PCR-fingerprinting experiments with the same three primers were carried out with a larger set of *H. jecorina* strains (14 strains from different geographical origins) and *T. reesei* QM6a. One strain each of *T. longibrachiatum*, *H. schweinitzii*, and *T. pseudokoningii* was included to illustrate the homology of the *H. jecorina*/*T. reesei* group in comparison to other species (data not shown). The strict consensus of 33 most parsimonious trees generated by parsimony analysis based on these PCR-fingerprinting results is shown in Fig. 3. Weighting of PCR-fingerprinting bands (including zero weight for faint bands) did not have significant effect on the parsimony tree topology. All *H. jecorina* strains and *T. reesei* form a common, but poorly resolved, clade, which is supported by 100% in bootstrap analysis. Additionally phenetic analysis using the UPGMA and Fitch–Margoliash methods (30, 31) was carried out, which yielded essentially the same results (data not shown).

DISCUSSION

While all species of *Trichoderma* sect. *Longibrachiatum* or the *Hypocrea* species with anamorphs assigned to this section have a characteristic morphology indicating close overall interrelatedness, there is no morphological feature than can lead to the conclusion that some species are more closely related than others. Molecular approaches can give phylogenetic insight into these relationships.

ITS sequences of *T. reesei* and *H. jecorina* are identical. Sequences of other species of sect. *Longibrachiatum* as well as of *H. schweinitzii* are different from each other and from *T. reesei*/*H. jecorina*, ranging from 2.2–4.8% for ITS-1 (Table 2). Among the various species studied, we found maximum intraspecific variation of 0.9% for ITS-1. In no case did different species show identical ITS sequences. The variation among the species of sect. *Longibrachiatum* is low if compared with most published data on intraspecific and interspecific variation of the ITS region in fungi (35–39). Intraspecific variations of the ITS sequences between 1 and 15% have been observed in diverse fungi, including asco- and basidiomycetes (35–39). Taken together, published data demonstrate an overlapping of the intra- and interspecific variation and ITS sequences should therefore not be used on their own for taxonomic conclusions at or below species level.

However, sequence analysis of one of the most variable regions within the ribosomal DNA gene complex (ITS) and PCR-fingerprinting data argue that *T. reesei* and *T. longibrachiatum* are taxonomically separable despite close morphological and biochemical similarity. These results are in agreement with other observations including data obtained by DNA-fingerprinting (15), investigation of cellulase genes (14), isozyme analyses (16, 17), and colony characteristics and growth rate (16).

The separation of the tropical *H. jecorina* from the temperate *H. schweinitzii* both by PCR-fingerprinting and sequence data agree with previous observations based on morphology and isozymes (16). The ITS sequences of *H. jecorina* strains were identical, indicating that there is considerable genetic continuity within this pantropical species. PCR-fingerprinting patterns derived from the pantropical collections of *H. jecorina* in the present study and isozyme results derived from a smaller number of tropical American collections (16, 17) indicated little divergence in *H. jecorina*. In this paper, the combined results of PCR-fingerprinting and DNA sequencing indicate that *T. reesei* and *H. jecorina* are not only closely related, as was suggested by isozyme analysis (17), but that they are likely to be the same species.

T. reesei and *H. jecorina* differ in phenotypic characters (16). Our interpretation of this observation is that *T. reesei* has been derived from a population of *H. jecorina* and that minor mutations have led to differing phenotypic manifestations. Unfortunately, because *T. reesei* is known only from one isolate, we have no idea of variability within the species and cannot rule out the possibility that the phenotypic characters and apparent loss of sexuality have developed after more than 50 years of laboratory cultivation. Our results are consistent with the assumption of a process whereby a population of *H. jecorina* in the Solomon Islands lost the ability for sexual reproduction and then underwent mutations that are, in part, reflected in PCR-fingerprinting, isozyme, and cultural characters. It is likely that *H. jecorina* also occurs in the Solomon Islands, and it is at least conçeivable that a derivative of *H. jecorina,* now known as *T. reesei,* was collected during a wartime investigation of the deterioration of canvas.

The identical ITS sequences, the similar PCR-fingerprints, and the comparable abilities to produce large quantities of cellulolytic enzymes (40) suggest a high level of genomic similarity between *H. jecorina* and *T. reesei. H. jecorina* is heterothallic (16), but we did not observe any indication of sexual crossing when *T. reesei* was mated with any of our strains of *H. jecorina*. While we cannot prove reproductive isolation or sexual incompetence for *T. reesei*, its failure to produce perithecia in crosses with *H. jecorina* supports the idea of reproductive isolation. The possibility of a multiple independent loss of sexuality to give anamorph groups was proposed for the ascomycetes *Talaromyces* (20) and *Epichloë* (41).

The identification of *H. jecorina* as the teleomorph of *T. reesei* provides means to study formation by classical genetics of cellulases and other enzymes by *H. jecorina*. Interestingly, several *H. jecorina* strains produced larger quantities of cellulases than the original *T. reesei* isolate QM6a (40). The apparent genetic proximity between *T. reesei* and *H. jecorina* also provides a system for the isolation and study of mating-type genes in *Trichoderma.*

The combined use of sequence analysis of the ITS regions of the rDNA and the more sensitive PCR-fingerprinting technique has documented for the first time the derivation of an apparently strictly asexual fungus, an anamorphic holomorph, from a sexual species. Previously anamorphic holomorphs have been more broadly linked to sexual genera (20) through multiple loss of sexuality or have been shown to be sterile hybrids of two sexual species (41, 42).

However, *T. reesei* and *H. jecorina* are separable because of differing isozyme profiles and phenotypic differences (16). These results have serious implications for taxonomy of deuteromycetes generally. The visible differences between the two are no more or no less than the visible differences between either *T. longibrachiatum* or *Trichoderma citrinoviride*. In the absence of any molecular information, *T. reesei* could easily be taken to be a biological species. Perkins (3) questioned whether the term "species" can even be applied to anamorphic fungi because they are not simply reproductively isolated, but are apparently incapable of sexual reproduction and consequent genetic recombination through meiosis and thus exist as clonal lines. The relationship between *H. jecorina* and *T. reesei* represents a phenomenon that might be common in nature. Given the seemingly endless number of 'species' of economically important deuteromycete genera such as *Alternaria*, *Penicillium*, *Trichoderma*, *Fusarium*, or *Phoma*, one can only wonder how many, or which ones, represent phenotypic variants that are otherwise genetically indistinguishable from others. A real dilemma highlighted by Seifert *et al.* (43) is that while phenotypic characters might not accurately account for species diversity, phenotype is still the primary tool for species recognition. DNA-based techniques have made possible the integration of asexual and sexual fungi at the generic level (44, 45). We have demonstrated that possibility at the species level for an individual anamorph/teleomorph pair. Because our results imply that any sexual population is capable of generating one or more asexual clones (?species), it is unlikely that integration at the species level will be achieved for more than selected cases.

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